

# Effects of activated protein C on the mesenteric microcirculation and cytokine release during experimental endotoxemia

*[Effets de la protéine C activée sur la microcirculation mésentérique et la libération de cytokines durant une endotoxémie expérimentale]*

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**Purpose:** Activated protein C (APC) is the first anti-inflammatory drug to be approved for the treatment of severe sepsis. However, the underlying mechanisms are not completely elucidated. Therefore, the aim of our study was to evaluate the effects of APC on the microcirculation (mesenteric leukocyte-endothelial interaction, plasma extravasation) using intravital microscopy (IVM) and on cytokine release during experimental endotoxemia in rats.

**Methods:** We divided forty, male, Lewis rats into four groups ( $n = 10$  per group): Controls, LPS (15 mg·kg<sup>-1</sup> lipopolysaccharide iv), APC (2 mg·kg<sup>-1</sup> APC iv), and LPS+APC. We determined mesenteric leukocyte-endothelial interactions and plasma extravasation at zero, one and two hours following administration of LPS and APC by IVM. Plasma levels of tumour necrosis factor- $\alpha$ , IL-1 $\beta$ , interleukin (IL)-6, and IL-10 were measured at zero and at two hours.

**Results:** Leukocyte adherence (-74%) and plasma extravasation (-28%) during endotoxemia were diminished significantly following APC treatment, compared to untreated LPS animals ( $P = 0.0001$  and  $P = 0.0004$ , respectively). Interleukin-1 $\beta$  release was also significantly reduced by APC treatment ( $2567.4 \pm 320.9$  pg·mL<sup>-1</sup> in the LPS group vs  $1626.1 \pm 427.2$  pg·mL<sup>-1</sup> in the LPS+APC group;  $P = 0.001$ ).

**Conclusion:** These rodent experiments showed that APC treatment significantly attenuated deterioration of the mesenteric microcirculation and systemic IL-1 $\beta$  release caused by endotoxin challenge. Because of the crucial role of the microcirculation in ongoing sepsis pathogenesis and multiple organ dysfunction syndrome, these effects may be of clinical importance.

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**Objectif :** La protéine C activée (PCA) est le premier médicament anti-inflammatoire à être approuvé pour le traitement des septicémies sévères. Cependant, les mécanismes sous-jacents de cette protéine ne sont pas encore complètement compris. C'est pourquoi notre étude avait pour but d'évaluer les effets de la PCA sur la microcirculation (interaction mésentérique leucocytaire-endothéliale, extravasation plasmatique) en utilisant la microscopie intravitale (IVM) ainsi que ses effets sur la libération de cytokines pendant une endotoxémie expérimentale chez les rats.

**Méthode :** Nous avons randomisé quarante rats Lewis mâles en quatre groupes ( $n = 10$  par groupe) : témoin, LPS (15 mg·kg<sup>-1</sup> lipopolysaccharide iv), PCA (2 mg·kg<sup>-1</sup> PCA iv), et LPS+PCA. Nous avons évalué les interactions mésentériques leucocytaires-endothéliales et l'extravasation plasmatique à zéro, une et deux heures après l'administration de LPS et de PCA par MIV. Les niveaux plasmatiques de facteur onconécrosant- $\alpha$ , d'IL-1 $\beta$ , d'interleukine (IL)-6, et d'IL-10 ont été mesurés à zéro et deux heures.

**Résultats :** L'adhérence leucocytaire (-74 %) et l'extravasation plasmatique (-28 %) pendant l'endotoxémie ont été significativement réduites après un traitement avec PCA, par rapport aux animaux LPS non traités ( $P = 0,0001$  et  $P = 0,0004$ , respectivement). La libération d'interleukine-1 $\beta$  était également réduite de façon significative par le traitement avec PCA ( $2567,4 \pm 320,9$  pg·mL<sup>-1</sup> dans le groupe LPS vs  $1626,1 \pm 427,2$  pg·mL<sup>-1</sup> dans le groupe LPS+PCA ;  $P = 0,001$ ).

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**Conclusion :** Ces expériences sur les rongeurs ont montré que le traitement à base de PCA atténuait de manière significative la détérioration de la microcirculation mésentérique ainsi que la libération systémique d'IL-1 $\beta$  provoquées par le choc endotoxique. En raison du rôle primordial de la microcirculation dans la pathogenèse continue de la septicémie et dans le syndrome de défaillance multi-systémique, ces effets pourraient avoir une importance clinique.

**S**EPSIS, severe sepsis, and septic shock are the most frequent causes of death in surgical, intensive care unit patients.<sup>1</sup> These sepsis syndromes represent significant cost to the health care system.<sup>2</sup> While there are significant advances in understanding the pathophysiology of sepsis, the search for a “cure” and a “management strategy” to treat sepsis remains. Because of the complex pathomechanisms, different strategies may be of potential benefit. Antibiotics are able to reduce the bacterial burden. In addition, circulating toxins and mediators may be blocked by specific antibodies. Further downstream in the pathophysiology, specific inhibitors (e.g., radical scavengers) may protect cells and tissues from the action of terminal mediators.

In sepsis, with full-blown systemic inflammation, strategies have been developed to attenuate these changes and to improve survival. Activated protein C (APC) is the first such drug to be approved for the treatment of severe sepsis.<sup>3</sup> Natively involved in the coagulation system, this substance exerts significant anti-inflammatory action. Activated protein C is converted from its inactive precursor, protein C, by thrombin coupled to thrombomodulin.<sup>4</sup> The activation of protein C is impaired during sepsis, secondary to the down-regulation of thrombomodulin by inflammatory cytokines.<sup>5</sup> Administration of human, recombinant APC has been shown to be effective in treating established, systemic inflammation.

Although cross-links between coagulation and inflammation were well-known, even before the large PROWESS trial,<sup>3</sup> the underlying mechanism of the beneficial action of APC during sepsis is not yet completely understood. In a previous study, we evaluated the effects of APC within the intestinal microcirculation during experimental endotoxemia.<sup>6</sup> The intestine is a pathophysiological vital organ and is considered to be the “motor of multiple organ failure” in sepsis.<sup>7</sup> However, distinct parameters of the microcirculation, such as plasma extravasation, were not studied within the intestinal wall for methodological reasons. Therefore, the aim of the present study was to evaluate the effects of APC within the mesenteric microcirculation,

including plasma extravasation using intravital microscopy (IVM), as well as to evaluate its impact on the release of the cytokines tumour necrosis factor (TNF)- $\alpha$ , interleukin (IL)-1 $\beta$ , IL-6 and IL-10 during experimental endotoxemia in rats.

## Methods

### Animals

After obtaining approval from the Institutional Animal Care Committee, we used 40, male, Lewis rats in the experiments (body weight: 250  $\pm$  50 g, Charles River Laboratories, Sulzfeld, Germany). We treated all animals in accordance with the standards and procedures set forth by the German animal safety legislations and by the Canadian Council on Animal Care. The animals were housed under 12 hr light/dark cycles, with temperature kept at 22°C and humidity at 55–60%. Standard diet and water were available *ad libitum*. No animal died during the experiment. After the experiment, the animals were killed by an overdose of intravenous pentobarbital.

### Anesthesia and preparation

The detailed, experimental procedure was previously described by the authors.<sup>8</sup> Briefly, for induction of anesthesia, we administered 60 mg·kg<sup>-1</sup> pentobarbital *ip* (Synopharm, Barsbüttel, Germany); and we maintained anesthesia with repeated injections of 5 mg·kg<sup>-1</sup> pentobarbital *iv*. Although, for the duration of the experiment, the animals spontaneously breathed room air, all animals received a tracheostomy to permit airway access. We introduced polyethylene catheters (PE 50, internal diameter 0.58 mm, external diameter 0.96 mm, Portex, Hythe, Kent, UK) into the left, external jugular vein and the right, common carotid artery. Arterial blood pressure and heart rate (HR) were continuously monitored and recorded during the study (Hewlett Packard monitor, Model 66S, Saronno, Italy). In order to study the microcirculatory effects of endotoxemia, without influences of hypotension, an adequate endotoxin dosage was identified in previous pilot experiments. Continuous body temperature was maintained at 37  $\pm$  0.5°C by use of a heating pad. Subsequently, median laparotomy was performed. A 15 min resting period followed.

### General protocol

We divided the animals into four groups ( $n = 10$  per group). We induced endotoxemia in two groups by administration of 15 mg·kg<sup>-1</sup> lipopolysaccharide (LPS) *iv* from *Escherichia coli*, serotype O111:B4 (Sigma-Aldrich, Steinheim, Germany). The two control groups received an equivalent amount of saline.

Immediately following endotoxin or saline administration, ten animals from each group received 2 mg·kg<sup>-1</sup> APC (Drotrecogin alpha (activated) - Xigris®, Lilly, Germany; all agents were given intravenously). To be consistent with reference literature and our previous experiments, APC was given as a bolus instead of an infusion.<sup>6,9</sup>

#### *Intravital fluorescence microscopy*

We performed intravital fluorescence microscopy at zero, one, and two hours following endotoxin/placebo (saline) administration (for details of the experimental settings please refer to our previous study).<sup>10</sup> The examination was directed to one venule of the mesentery of the terminal ileum. A supporting device held the intestine in place. A cover slip served as a transparent cover of the mesentery. Using this method, approximately 1 cm<sup>2</sup> of the mesentery could be evaluated by microscopy. Intravital microscopy equipment consisted of: the epifluorescent microscope, Axiotech Vario (Carl Zeiss, Jena, Germany); a light source, HBO 50 (Carl Zeiss); oculars 10 x (Carl Zeiss); lens 20x/0.5, Achroplan (Carl Zeiss); a filter type #20 (Carl Zeiss), for evaluation of leukocyte-endothelial interactions; a filter type #10 (Carl Zeiss), for examinations of plasma extravasation; a black and white-CCD video camera (BC-12, AVT-Horn Aalen, Germany); a S-VHS video tape recorder (Panasonic NV-SV120EG-S, Matsushita Audio Video, Germany); and a black and white monitor (PM-159, Ikegami Electronics, Germany). We achieved a total magnification of 500X at the 14-inch monitor, with this configuration.

#### *Leukocyte-endothelial interaction*

Leukocytes were stained *in vivo*, by injection of 0.2 mL *iv* of 0.05 g % rhodamine 6G (MW 479, Sigma, Deisenhofen, Germany), for contrast enhancement 15 min before IVM start.<sup>11</sup> Rhodamine 6G stains all types of leukocytes. Therefore, it was not possible to differentiate which subtypes were adherent and/or whether this changed with time. Rollerflow was given as the number of nonadherent, rolling leukocytes passing through the observed vessel segment within 30 sec. We defined adherent leukocytes (stickers) in each vessel segment as cells that did not move, or did not detach from the endothelial lining within an observation period of 30 sec. "Stickers" were given as the number of cells per mm<sup>2</sup> of endothelial surface, calculated from diameter and length of the vessel segment studied, assuming cylindrical geometry. The evaluation of leukocyte adherence was performed in a blinded fashion.

#### *Plasma extravasation*

To quantify plasma extravasation across mesenteric venules, we injected 50 mg·kg<sup>-1</sup> FITC-BSA (Sigma, Deisenhofen, Germany) ten minutes before the experiment.<sup>12</sup> The recorded, fluorescent images were digitized; and the gray levels, depending on the fluorescence activity (gray levels range from 0 [black] to 255 [white]), were measured within five segments of the venule under study (Iv) and in five contiguous areas of the perivenular interstitium (Ip). Plasma extravasation (macromolecular leakage) was expressed as the ratio Ip/Iv after one hour of endotoxemia. The evaluation was performed in a blinded fashion.

#### *Laboratory analysis*

We drew arterial blood (0.55 mL), at the time point zero and two hours, for blood gas and hematocrit analysis (ABL 330, Radiometer, Hamburg, Germany). In addition, 280 µL of plasma were fractionated for cytokine analysis (TNF-α, IL-1β, IL-6, and IL-10 Rat-Quantikine ELISA kits, R&D Systems, Wiesbaden, Germany).

#### *Statistics*

We determined the required sample size, before the experiments for the primary endpoint parameters, leukocyte adherence, and plasma extravasation. The minimal sample size, to detect a significant treatment effect, was calculated with  $n = 10$ . We performed data analysis non-parametrically with a statistical software package (SPSS 15.0 for Windows, SPSS Inc., Chicago IL, USA). All data were analyzed using a one-way analysis of variance, followed by the Newman-Keuls multiple comparison test. Mean arterial pressure (MAP) and HR were analyzed by a two-way analysis of variance (ANOVA) (repeated measures in the factor of time), followed by the Newman-Keuls multiple comparison test when ANOVA was significant. A  $P < 0.05$  value was considered significant.

#### **Results**

In the control animals, HR and MAP did not change during the observation period (Figures 1A and B). Lipopolysaccharide (LPS) administration was associated with a significantly elevated HR. Heart rate in APC treated animals increased initially, comparable to untreated LPS animals. At the end of the observation period, only the HR of animals in the untreated LPS group was significantly elevated, in comparison to control group. Activated protein C treatment, with or without endotoxemia, was found to decrease MAP. However, MAP values of all groups remained within the animals' physiological range.

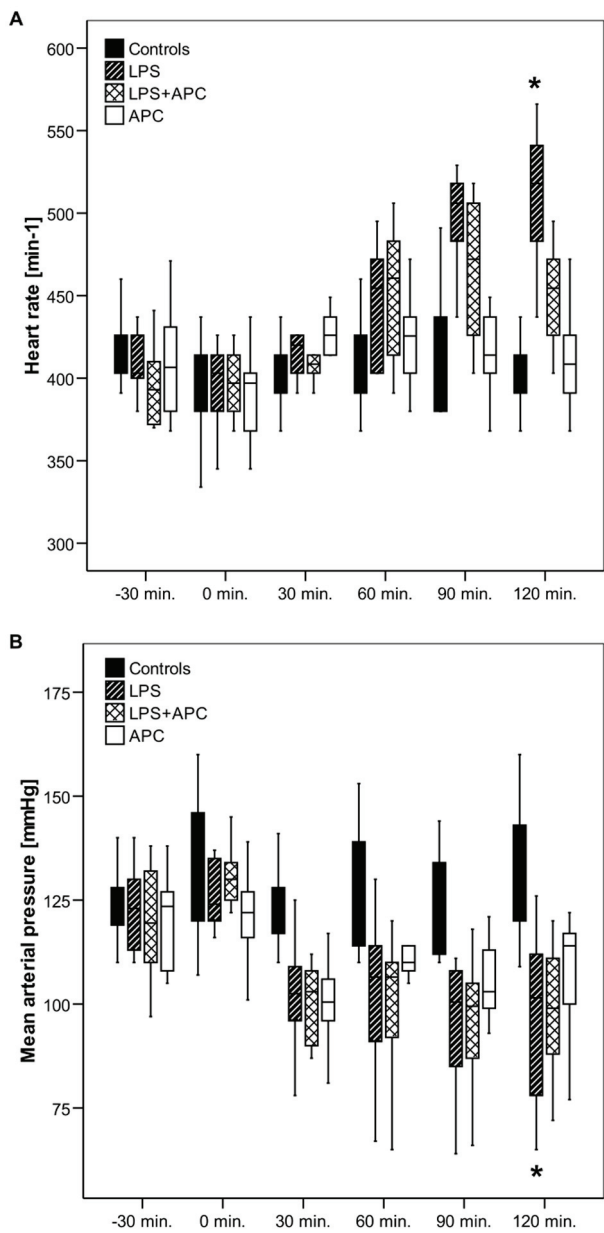


FIGURE 1 Panel A: Heart rate (min<sup>-1</sup>) - significant difference at 120 min lipopolysaccharide (LPS) group *vs* lipopolysaccharide + activated protein C (LPS+APC) group (*P* < 0.05; median, 10<sup>th</sup>, 25<sup>th</sup>, 75<sup>th</sup>, 90<sup>th</sup> percentiles). Panel B: Mean arterial pressure (mmHg) - significant differences at 120 min LPS, APC, and APC+LPS groups *vs* control group (*P* < 0.05; median, 10<sup>th</sup>, 25<sup>th</sup>, 75<sup>th</sup>, 90<sup>th</sup> percentiles).

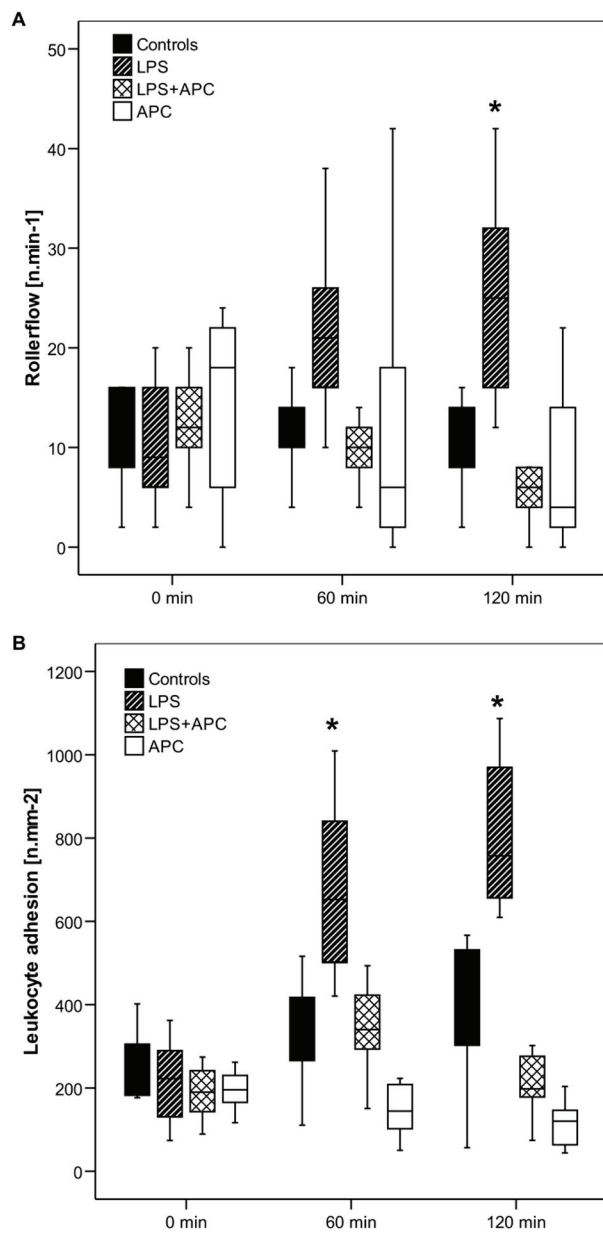


FIGURE 2 Panel A: Rollerflow (n.min<sup>-1</sup>) - significant difference at 120 min (third intravital microscopic examination) lipopolysaccharide (LPS) group *vs* lipopolysaccharide + activated protein C (LPS+APC) group (*P* < 0.05; median, 10<sup>th</sup>, 25<sup>th</sup>, 75<sup>th</sup>, 90<sup>th</sup> percentiles). Panel B: Leukocyte adhesion (n.mm<sup>-2</sup>) - significant difference at 60 min (second intravital microscopic examination) and 120 min (third intravital microscopic examination) LPS group *vs* LPS+APC group (*P* < 0.05; median, 10<sup>th</sup>, 25<sup>th</sup>, 75<sup>th</sup>, 90<sup>th</sup> percentiles).

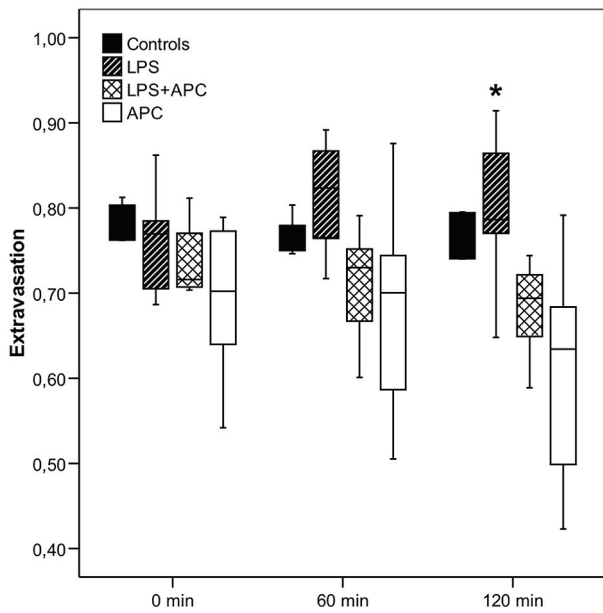


FIGURE 3 Plasma extravasation (%) – significant difference at 120 min (third intravital microscopic examination) lipopolysaccharide (LPS) group *vs* lipopolysaccharide + activated protein C (LPS+APC) group ( $P < 0.05$ ; median, 10<sup>th</sup>, 25<sup>th</sup>, 75<sup>th</sup>, 90<sup>th</sup> percentiles).

Figures 2A and 2B show the leukocyte-endothelial interactions during the experiment, as observed by IVM. The rolling behaviour (rollerflow) was unchanged in the control group during the experiment. Rollerflow significantly increased following endotoxin challenge. Activated protein C treatment completely reversed this behaviour. In the untreated LPS group, there was a significant increase in the number of sticking leukocytes in the venules (leukocyte adhesion). Activated protein C treatment attenuated leukocyte adherence (-74%;  $P = 0.0001$ ).

Changes in the plasma extravasation during the experiment are shown in Figure 3. In the untreated LPS group, there was a slight, but significant, increase in plasma extravasation during the observation time. Activated protein C administration decreased the plasma extravasation significantly, compared to untreated LPS animals (-28%,  $P = 0.006$ ) (see Figure 3).

The results of blood gas and hematocrit analysis did not differ between endotoxemic and control animals, except for slight hypocapnia, due to hyperventilation following LPS administration (data not shown). Endotoxin challenge resulted in a significant systemic release of inflammatory cytokines, compared to con-

TABLE Cytokine levels

	Control	LPS	LPS+APC	APC
TNF - $\alpha$	17.24 (9.5– 34.02)	* 921.83 (752.47 – 1146.24)	* 1242.63 (1049.82 – 1330.88)	64.38 (51.51 – 69.63)
IL - 1 $\beta$	318.12 (125.43 – 540.26)	* 2238.06 (1982.49 – 3600.01)	§ 1974.36 (115.26 – 2375.79)	252.89 (125.49 – 298.30)
IL - 6	418.94 (331.57 – 953.19)	* 11639.04 (11565.01 – 11739.39)	* 11618.38 (11569.21 – 11666.44)	514.24 (353.50 – 576.14)
IL - 10	147.82 (120.09 – 211.34)	* 720.02 (446.14 – 1170.99)	* 1018.76 (490.38 – 1352.03)	270.69 (204.92 – 669.88)

Median (25<sup>th</sup>, 75<sup>th</sup> percentiles); cytokine levels ( $\text{pg}\cdot\text{mL}^{-1}$ ) following two hours of observation. LPS = lipopolysaccharide; LPS + APC = lipopolysaccharide + activated protein C; TNF- $\alpha$  = tumour necrosis factor  $\alpha$ ; IL = interleukin. \* $P < 0.05$  *vs* control, § $P < 0.05$  *vs* control and LPS.

trol values (Table). Activated protein C treatment significantly attenuated the release of IL-1 $\beta$ . Tissue release of TNF- $\alpha$ , IL-6, and IL-10 was unaffected.

**Discussion**

In the present study, APC treatment appeared to attenuate deterioration of the mesenteric microcirculation during experimental endotoxemia in rats, by significantly reducing leukocyte adherence. We were able to demonstrate, for the first time, that endotoxin-induced, plasma extravasation can be attenuated by APC treatment. Activated protein C treatment decreased the systemic release of the cytokine IL-1 $\beta$ .

The beneficial action of APC in sepsis seems to be closely related to its effects within the microcirculation. In 1994, Grinnell *et al.*<sup>13</sup> showed that human plasma-derived and human cell-produced recombinant protein C inhibits E-selectin mediated cell adhesion to the vascular endothelium. Activated protein C also attenuated endotoxin-derived, pulmonary vascular injury in rats, by inhibiting activated leukocytes.<sup>14</sup>

Three recently published studies investigated the effects of the APC on microcirculation, during experimental endotoxemia by intravital fluorescence microscopy.<sup>6,9,15</sup> Hoffmann *et al.*<sup>15</sup> reported that the APC diminishes endotoxin-derived reduction of functional capillary density, as well as leukocyte adherence to the endothelium in dorsal, skinfold chamber preparation. In a previous study, our group found similar results in

the intestinal wall.<sup>6</sup> Iba *et al.*<sup>9</sup> described a suppression of leukocyte adhesion in the mesentery, following APC treatment during experimental endotoxemia. In this study, we confirmed the findings of Iba *et al.*<sup>9</sup> using a much higher endotoxin load. Additionally, we demonstrated that the APC administration could reduce plasma extravasation within the mesenteric microcirculation.

Leukocyte adhesion to the endothelium is a typical finding during experimental endotoxemia and sepsis. Visualization of leukocyte-endothelial interactions is a domain of intravital, fluorescence microscopy. Initially, leukocytes marginate from the centerline of the blood stream. Leukocyte rolling along the endothelium is mediated by selectins.<sup>16</sup> Integrins mediate the following step of leukocyte activation with firm adherence, known as leukocyte sticking, to the endothelial wall.<sup>17</sup> Emigration into tissue represents the final step and is mediated in local inflammation by chemokines and other mediators.<sup>18</sup> In systemic inflammation, the activation, adherence, and emigration of leukocytes in various organs leads to a disturbance of microcirculation, followed by multiple organ dysfunction syndrome (MODS) and multiple organ failure (MOF). In the microcirculation of septic rat skeletal muscle, Piper *et al.*<sup>19</sup> investigated leukocyte activation and microvascular flow. Within the first 24 hr after sepsis induction, leukocyte adhesion increased. However, in parallel to the reduction of circulating white blood cell count, leukocyte adhesion returned to baseline. Therefore, leukocyte adhesion is only one factor responsible for impairment of the microcirculatory blood flow.

Plasma extravasation represents a relevant feature during sepsis. It is the result of the “capillary leakage syndrome” and leads to further deterioration of the microcirculation, because of tissue edema and hypotension caused by relative hypovolemia.<sup>12</sup> Eventually, plasma extravasation contributes to MODS and MOF in septic patients. Consequently, attenuation of plasma extravasation by an agent may be of clinical importance.

In our experiments, APC treatment appeared to reduce plasma extravasation, even in the absence of endotoxemia. It is possible that there was an initial increase in plasma extravasation (time point zero hours) following preparation of the mesentery. This would, in part, explain the only slight, but significant, increase of plasma extravasation following endotoxin challenge. However, APC administration decreased plasma extravasation significantly in animals, both with and without endotoxemia.

Another factor responsible for impairment of the microcirculation is coagulation activation. In addition to improving the survival of patients with sepsis, the

APC also has anticoagulatory effects. This is in contrast to other potent inhibitors of coagulation, such as Antithrombin III, which failed to reduce mortality in septic patients.<sup>20</sup> In summary, APC exerts multifactorial actions on microvascular distress, which are closely linked to anti-inflammation and anti-coagulation.<sup>21</sup>

Several animal studies showed that APC treatment, in inflammatory conditions, reduces the release of inflammatory cytokines. For example, Mizutani *et al.*<sup>22</sup> reported that APC significantly inhibited ischemia/reperfusion-induced increase of TNF- $\alpha$  and IL-8 and, thereby, prevented pulmonary vascular injury. Although the APC attenuated endotoxin-induced IL-1 $\beta$  release in this study, it did not have any effects on the TNF- $\alpha$ , IL-6, and IL-10 levels. Iba *et al.*<sup>9</sup> observed a significant reduction of TNF- $\alpha$  levels, by APC treatment, following one hour of endotoxemia – at the time of the TNF- $\alpha$  release peak. At the three-hour time point, this effect was not significant in endotoxemic animals treated with 5 mg·kg<sup>-1</sup> APC. It is possible that the timing of our experimental protocol did not allow tracking of the TNF- $\alpha$  (and IL-6/IL-10) levels at maximum release. However, intravenous administration of recombinant, human-activated protein C in rats with *P. aeruginosa* pneumonia did not have effects on neutrophil influx and activity or expression of pulmonary cytokines.<sup>23</sup> Interspecies, interstrain and model variabilities must be considered in the interpretation of experimental results. With regard to LPS challenge, Jesch *et al.*<sup>24</sup> found significant differences in macrophage response (formation of nitric oxide) between five investigated rat strains. Our previously reported data (using a different strain of rats, due to a change in supplier) showed a greater than tenfold difference in several of the cytokine levels.<sup>6</sup> In addition, the hemodynamic response may vary, depending on the investigated rat strain. While Deschepper *et al.*<sup>25</sup> conducted experiments in mice to systematically review interstrain variance for quantitative traits in cardiovascular responses, including systolic blood pressure, no comparable studies are available in rats. In our experiments, we found significantly different hemodynamic conditions in response to LPS, with similar settings but different rat strains.<sup>6</sup>

Other limitations of our study include induction of a sepsis-like condition by intravenous administration of endotoxin. This differs from clinical circumstances and the administration of a single bolus of APC, which differs from a continuous, zero-order infusion of APC ( $\mu\text{g}\cdot\text{kg}^{-1}\cdot\text{hr}^{-1}$ ) used in the PROWESS study.<sup>3</sup> In addition, the underlying mechanism of APC action on cytokine release could not be elucidated in our experimental setting.

In summary, the aims of our study were to evaluate the effects of APC, within mesenteric microcirculation using IVM, and to measure the systemic release of cytokines TNF- $\alpha$ , IL-1 $\beta$ , IL-6, and IL-10, during experimental endotoxemia in rats, to elucidate mechanisms of anti-inflammatory action. Our experiments showed that APC treatment significantly attenuated the deterioration of the mesenteric microcirculation (leukocyte adherence and plasma extravasation) and the systemic IL-1 $\beta$  release. Because of the microcirculation's crucial role in the pathogenesis of ongoing sepsis and multiple organ dysfunction syndrome, the beneficial effects of APC may play an important role in the management of patients with sepsis.

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